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Award Number: W81XWH-09-1-0385

TITLE: Highly specific targeting of the TMPRSS2/ERG fusion gene in prostate cancer using liposomal nanotechnology

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REPORT DATE: June 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE June 2013		2. REPORT TYPE Final		3. DATES COVERED 15 May 2009 – 14 May 2013	
4. TITLE AND SUBTITLE Highly specific targeting of the TMPRSS2/ERG fusion gene in prostate cancer using liposomal nanotechnology				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-1-0385	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bulent Ozpolat, M.D., Ph.D. Michael Ittmann, M.D., Ph.D. E-Mail: mittmann@bcm.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas M.D. Anderson Cancer Center Houston, TX 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
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15. SUBJECT TERMS None provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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DOD grant : W81XWH-09-1-0385,

Title: "HIGHLY SPECIFIC TARGETING OF THE TMPRSS2/ERG FUSION GENE IN PROSTATE CANCER USING LIPOSOMAL NANOTECHNOLOGY

ABSTRACT

The TMPRSS2/ERG fusion gene is found in about 55 % of prostate cancer (PCa) patients. It is absolutely specific for PCa cells, since the fusion transcript is only present in these cells. There is heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene. Some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We seek to target the four most common and biologically active alternatively spliced fusion gene transcript isoforms using SiRNAs to obtain maximal biological activity in cancers expressing a specific isoform or a combination of isoforms. We propose to use of systemically administered nanolipsosomal siRNAs specifically targeting the TMPRSS2/ERG mRNA fusion junctions in orthotopic prostate cancer model in mice. Because this fusion gene is highly specific to PCa we do not expect off-target effects in normal tissues or minimal toxicity. Our results support the efficacy of this approach in in vivo PCa models.

INTRODUCTION

Since its discovery use of small-interfering RNA (siRNA) has rapidly become a powerful tool for therapeutic and specific gene silencing. Recently siRNA technology has generated much excitement for possible use as a novel therapeutic modality. However, in vivo siRNA delivery has proven difficult because of lack of non-toxic and effective systemic delivery methods. We have developed neutral based nanolipsosomal delivery system for in vivo therapeutic use of siRNA therapeutics.

The discovery of recurrent fusion of the androgen-regulated TMPRSS2 gene to the ERG gene in the majority of prostate cancer (PCa) lesions, has led to a paradigm shift in the study of PCa. The TMPRSS2/ERG fusion gene occurs in 15-80% of PCa lesions, depending on the clinical stage, with 40-60% of surgically treated cancers containing the gene fusion. Most studies have shown an association between the presence of the TMPRSS2/ERG fusion and aggressive disease. We have now demonstrated that the TMPRSS2/ERG fusion gene isoforms can enhance proliferation, invasion and motility of prostate epithelial cells. More importantly, knockdown of the fusion gene in a cancer cell line inhibits tumor growth in vivo in an orthotopic mouse model, indicating that the TMPRSS2/ERG fusion gene is a potential therapeutic target which is present in the majority of prostate cancers.

All reports to date indicate that there is significant heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene. Thus, some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We have characterized 8 fusion types in PCa (1), which have been

confirmed by others. In all cases, the fusion mRNA includes the TMPRSS2 exon 1 and often exon 2, as well. The most common transcript contains the TMPRSS2 exon 1 fused to ERG exon 4, such that translation would have to arise from an internal ATG codon and give rise to a slightly truncated protein which we have designated as the Type III isoform. This variant is expressed in 86% of fusion gene expressing prostate cancers, either alone or in combination with other isoforms. Of particular interest is an isoform in which TMPRSS2 exon 2 is fused with ERG exon 4 (designated Type VI). This variant was present in 26% of our cases with fusion gene expression (1). For this isoform, translation can be initiated from the TMPRSS2 translation initiation codon and results in a true fusion protein containing the first five amino acids of the TMPRSS2 gene fused to a slightly truncated ERG protein. We found that expression of this isoform is associated with aggressive disease. Types I and II give rise to full length ERG protein arising from the native ERG ATG and are also associated with more aggressive disease. These isoforms are present in 20% and 11% of fusion gene expressing cancers respectively.

In vivo systemic siRNA delivery: The promise of specific RNA degradation has also generated much excitement for possible use as a novel therapeutic modality. However, successful application of siRNA therapeutics to clinic requires development of safe and effective delivery system. In vivo siRNA delivery has proven difficult because of lack of non-toxic and effective systemic delivery methods. We recently developed non-toxic neutrally charged 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC)-based liposomal nanovectors (mean size 65nm) that can target siRNA in vivo into tumor cells 10-fold and 30-fold more effectively than cationic lipids and naked siRNA, respectively, leading to significant and robust target gene silencing in orthotopic cancer models.

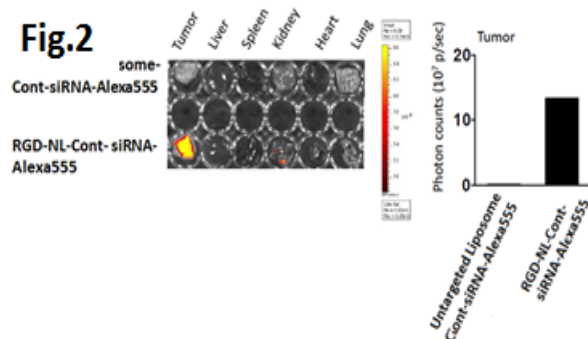
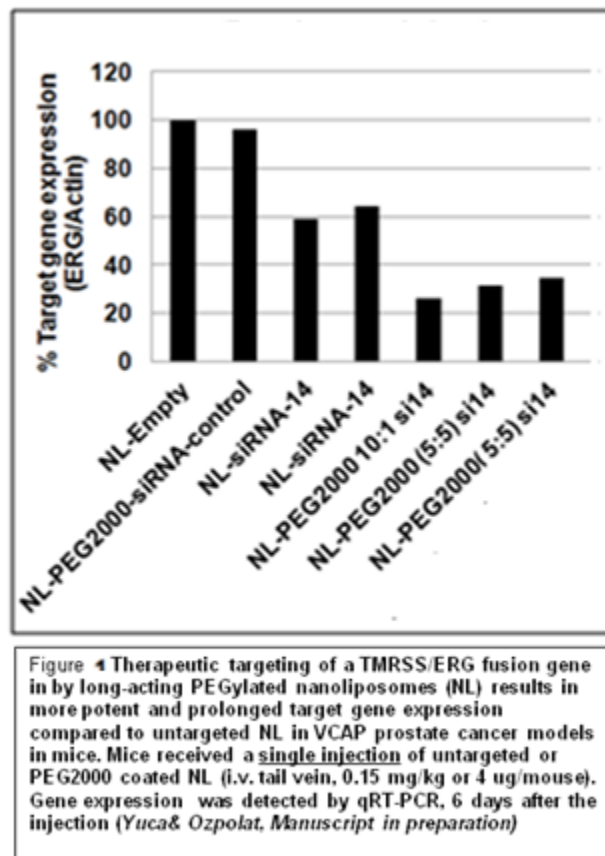
The TMPRSS2/ERG fusion gene is absolutely specific for prostate cancer cells, since the fusion transcript is only present in these cells. Unfortunately, there is heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene as described above. Thus, some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We seek to target the four most common and biologically active alternatively spliced fusion gene transcript isoforms, which constitute greater than 95% of all transcripts, to obtain maximal biological activity in cancers expressing a specific isoform or a combination of isoforms. In vivo knockdown of TMPRSS2/ERG fusion gene expression using liposomal nanovectors should decrease prostate cancer progression in vivo and be an effective therapeutic strategy in human prostate cancers bearing this fusion gene. Given the extremely high prevalence of this chromosomal alteration in human prostate cancer, the majority of prostate cancers may be amenable to this treatment. We propose to use siRNAs specifically targeting the TMPRSS2/ERG mRNA fusion junctions, which are present only in PCa cells, to minimize off-target effects in normal tissues so toxicity should be minimal.

No-Cost extension period

We have previously developed nanoliposomal siRNA incorporating siRNA targeting the junction of TMPRSS2/ERG and demonstrated that our nanoliposomal siRNA can significantly inhibit growth of prostate tumors in both orthotopic and subcutaneous xenografts in mice (Shao et al, clinical Cancer Res 2012). Because this strategy inhibited target gene expression no more than 40-45% we aimed to develop long circulating liposomal siRNA nanoparticles coated with PEG-2000. In this period we used long-circulating/acting PEGylated nanoliposomal siRNA to therapeutically silence the target fusion gene in animal models to determine if this approach significantly enhance the inhibition tumor growth in nude mice bearing human prostate tumors. We have found that PEGylated liposomes can silence the target gene at least 6 days after a single injection of siRNA injection in mice (siRNA 0.15 mg/kg, i.v, syatemic injection from tail vein).

Most important PEGlated liposomal siRNA (DOPC:DSPE-PEG2000; 10;1 and 5;5 ratios) was superior to regular DOPC-based liposomes in terms of better and robust target downregulation, leading to 70% target gene downmodulation in VCAP tumors-

Before combination with any therapy we aimed to further improve target gene downregulation we developed tumor targeting PEGylated liposomes conjugated high affinity ligands, such as RGD, folate, IGFR1 binding peptide to PEG-2000 arm that is bound to DSPE. We first tried this strategy in integrin α V β 3 expressing SKOV3 tumors using RGD-peptied expressing liposomes. Although α V β 3 (the receptor for RGD) is expressed in angiogenic tumor endothelium some tumor such as SKOV3 express α V β 3. We used this tumor model as a positive control to test in vivo distribution of our tumor-targeted siRNA-nanotherapeutics coated with PEG2000 and high-affinity adhesive ligands such as RGD (Arg-Gly-Asp) cyclic peptide (a ligand for α V β 3). As shown in Fig.2 and significantly silenced the target genes *in vivo* at least 6 days after a single injection and accumulated



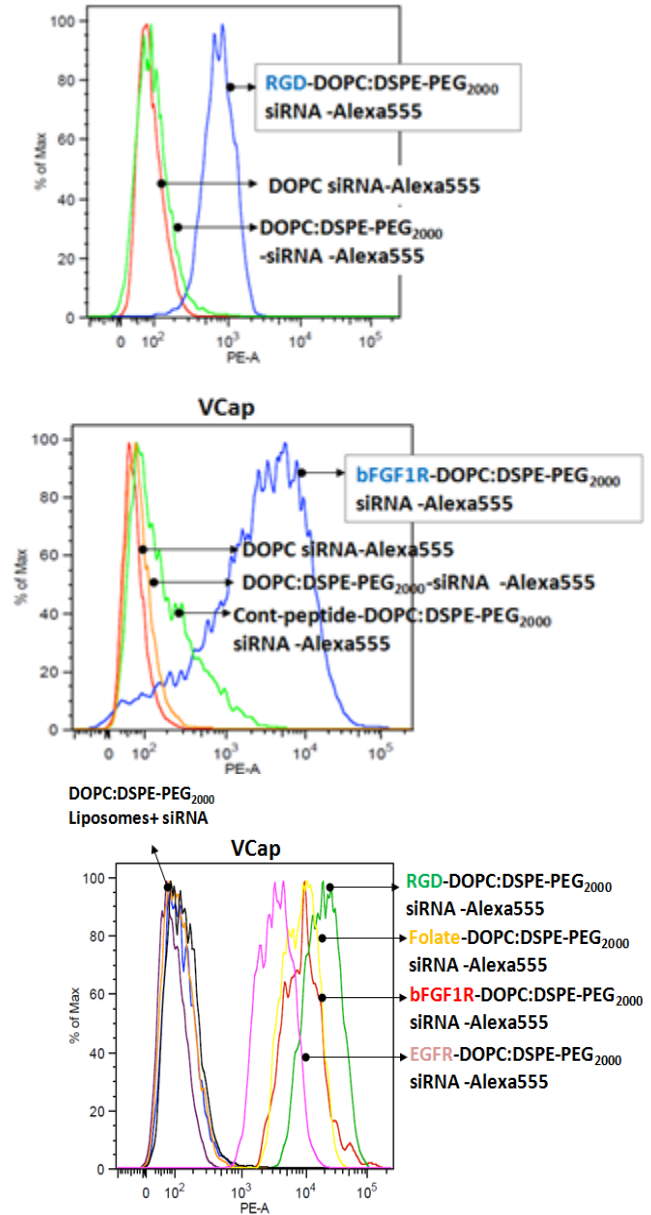
in tumors 14-fold more compared to untargeted liposomes (Fig. 2), suggesting that

After optimizing PEGylated nanoliposomes coated with PEG2000 attached to RGD, folate, IGFR1 binding peptide were tested in in vitro setting using VCAP prostate cell line. This tumor targeting strategy led to significantly increased in vitro siRNA uptake and into VCAP prostate cancer cells (Fig 3). During the period we also aimed to test efficacy of PEGylated siRNA nanotherapeutics targeting the TMPRSS20ERG fusion gene that we recently developed and optimized for use in animal models.

Zeta potential and charge of the liposomes were between -5 mV to 4 mV. Size of the liposomes were measured with zeta sized and ranged from 65 to 100u-

After optimizing PEGylated tumor-targeting long acting liposomes in vitro and in vivo settings we finally set to test these approached in mice bearing VCAP tumors. Current mice tumors are being analyzing mice tumors generated by injection with VCap cells. We are examining RGD-or IGFR coated peglated liposomes alone and in combination with chemotherapy to see in vivo target downmodulation and several markers of cell proliferation and apoptosis.

Fig.3



KEY RESEARCH ACCOMPLISHMENTS

- Developed high efficiency tumor targeting PEGylated nanoliposomes siRNA targeting the Type III fusion gene mRNA.

Tumor targeting nanoliposomes can we specifically targeted to tumors with much higher efficacy compared with normal liposomes.

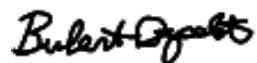
REPORTABLE OUTCOMES

- Developed long-acting Pegylated siRNA nanaovectors targeting tumor surface receptors and all the most common isoforms of the TMPRSS2/ERG fusion gene that lead to 70% target downmodulation for a prolonged period (~ 1 week) in prostate tumors.

CONCLUSION

Our results strongly support the concept that we can specifically target the TMPRSS2/ERG fusion gene in vivo using siRNAs and that this results in decreased tumor progression. We believe this is critical to successfully silence the fusion gene specifically expressed in majority prostate cancer patients. A manuscript reporting previous results was published *Clinical Cancer Research* (Shao et al 2012).

Respectfully,



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